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## Molecular insights into the progression of crystalline silica-induced pulmonary toxicity in rats

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### Abstract

Identification of molecular target(s) and mechanism(s) of silica-induced pulmonary toxicity is important for the intervention and/or prevention of diseases associated with exposure to silica. Rats were exposed to crystalline silica by inhalation (15 mg m<sup>-3</sup>, 6 h per day, 5 days) and global gene expression profile was determined in the lungs by microarray analysis at 1, 2, 4, 8 and 16 weeks following termination of silica exposure. The number of significantly differentially expressed genes (>1.5-fold change and <0.01 false discovery rate *P*-value) detected in the lungs during the post-exposure time intervals analyzed exhibited a steady increase in parallel with the progression of silica-induced pulmonary toxicity noticed in the rats. Quantitative real-time PCR analysis of a representative set of 10 genes confirmed the microarray findings. The number of biological functions, canonical pathways and molecular networks significantly affected by silica exposure, as identified by the bioinformatics analysis of the significantly differentially expressed genes detected during the post-exposure time intervals, also exhibited a steady increase similar to the silica-induced pulmonary toxicity. Genes involved in oxidative stress, inflammation, respiratory diseases, cancer, and tissue remodeling and fibrosis were significantly differentially expressed in the rat lungs; however, unresolved inflammation was the single most significant biological response to pulmonary exposure to silica. Excessive mucus production, as implicated by significant overexpression of the pendrin coding gene, *SLC26A4*, was identified as a potential novel mechanism for silica-induced pulmonary toxicity. Collectively, the findings of our study

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#### Microarray Data

The microarray data have been deposited in the Gene Expression Omnibus Database, <http://www.ncbi.nlm.nih.gov/geo> (accession number GSE32147).

#### Disclaimer

The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the NIOSH.

#### SUPPORTING INFORMATION

Supporting information can be found in the online version of this article.

provided insights into the molecular mechanisms underlying the progression of crystalline silica-induced pulmonary toxicity in the rat. Published 2012. This article is a US Government work and is in the public domain in the USA.

## Keywords

silica; pulmonary toxicity; gene expression; inflammation

## INTRODUCTION

Exposure to respirable crystalline silica (silica) takes place in a variety of industries and occupational settings because of its extremely common natural occurrence and the wide range of materials and products that contain it. Virtually any process that involves the movement of earth or disturbance of products such as concrete and masonry may expose workers to silica. It is estimated that at least 1.7 million workers in the USA and millions more worldwide are occupationally exposed to silica annually. Workers in several US industries are exposed to silica at concentrations much higher than the National Institute for Occupational Safety and Health (NIOSH) Recommended Exposure Level (Linch *et al.*, 1998). In addition to silicosis, a life-threatening lung pneumoconiosis, occupational exposure to silica is associated with the development of bronchitis, emphysema, tuberculosis, systemic sclerosis, rheumatoid arthritis, lupus, chronic renal disease and lung cancer (IARC, 1997; Cooper *et al.*, 2002). A proper understanding of the molecular targets and mechanisms underlying the initiation and progression of silica-induced pulmonary toxicity is required to develop strategies potentially to prevent the various diseases associated with silica exposure.

In spite of the large number of studies conducted in the past investigating the toxicity of crystalline silica, neither the molecular targets nor the mechanisms underlying its toxicity are fully understood. Advances in high-throughput gene expression profiling, such as microarray analysis, enable a comprehensive understanding of the effects of toxic agents at the molecular level in biological systems. Identification of the genes that are differentially expressed in a biological system in response to exposure to a toxic agent and careful bioinformatics analysis of the differentially expressed genes by employing appropriate statistical and computational approaches may provide valuable information regarding the molecular target(s) and/or the mechanism(s) underlying the toxicity of the agent. The gene expression data, in addition, may be useful to generate novel hypotheses regarding the molecular mechanisms underlying the toxicity of the agent being investigated. The differentially expressed genes and/or their products, following appropriate validation, may be employed as biomarker(s) for exposure as well as toxicity of the agent under investigation. In the past, microarray-based transcriptomics studies have been successfully employed to gain insights into the molecular mechanisms underlying the toxicity of chemicals (Waring *et al.*, 2001; Hamadeh *et al.*, 2002) as well as to identify molecular markers for their toxicities (Amin *et al.*, 2004; Fielden *et al.*, 2008). In addition, it has been fairly well established that gene expression changes relevant to toxicity precede biochemical and histological changes indicative of target organ toxicity (Foster *et al.*, 2007; Umbright *et*

*al.*, 2010). Since occupational exposure to silica might result in irreversible but preventable adverse health effects, for example silicosis, it is important not only to detect the early toxic effects of silica exposure but also to understand comprehensively the molecular targets and/or mechanisms underlying its toxicity.

Recently, we have reported developing a rat model for silica-induced pulmonary toxicity (Sellamuthu *et al.*, 2011). Inhalation exposure of rats to silica (15 mg m<sup>-3</sup>, 6 h per day, 5 days) resulted in the induction of pulmonary toxicity, which progressed steadily for several weeks after cessation of silica exposure. We determined the global gene expression profile in the lungs obtained from these rats to identify the molecular targets as well as to understand the mechanisms involved in silica-induced pulmonary toxicity progression. Bioinformatics analysis of the gene expression data identified molecular targets of silica toxicity and provided insights into the molecular mechanisms underlying the progression of silica-induced pulmonary toxicity in the rats.

## MATERIALS AND METHODS

### Animals

Approximately 3-month-old, pathogen-free male Fisher 344 rats (CDF strain), purchased from Charles River Laboratories (Wilmington, MA, USA), were used in the study. The rats upon arrival to our American Association for Assessment and Accreditation of Laboratory Animal Care International approved animal facility (NIOSH, Morgantown, WV, USA) were allowed to acclimatize for approximately 10 days prior to their use in this study. Two rats were housed per cage under controlled room temperature (22–25 °C) and humidity (40–60%). Throughout the period of the experiment the rats were maintained on a 12 h light–dark schedule with free access to rat diet (Harlan Laboratories, Frederick, MD, USA) and tap water.

### Chemicals

Min-U-Sil 5 silica (US Silica, Berkeley Springs, WV, USA) was used in this experiment. Detailed chemical composition of the silica sample used in our study is available at the supplier's website (<http://www.u-s-silica.com>). A thorough characterization of the silica particles generated and employed in this inhalation exposure study was performed. Representative samples collected from the inhalation exposure chamber were analyzed for particle morphology by scanning electron microscopy. Silica aerosol samples were also analyzed using a Micro-orifice Uniform Deposit Impactor (MOUDI) to determine particle size distribution. Results of these analyses can be found in our recent publication (Sellamuthu *et al.*, 2011). Commercial sources of all other reagents used in this study are provided in the corresponding sections below.

### Exposure of Rats to Silica Aerosol

The entire animal experiment was conducted following a protocol approved by the Institutional Animal Care and Use Committee (IACUC), NIOSH, Morgantown, WV, USA. Details regarding generation of the crystalline silica aerosol and inhalation exposure of rats to the aerosol have been published previously (Sellamuthu *et al.*, 2011). Stated briefly, an

aerosol containing crystalline silica particles of repairable size (mass median aerodynamic diameter = 1.6  $\mu\text{m}$ ) was generated from a bulk supply of Min-U-Sil 5 silica (US Silica, Berkeley Springs, WV, USA). A Venturi was placed in the inhalation exposure system in between the acoustic generator and the exposure chamber to prevent the agglomeration of silica particles generated. Forty rats were exposed to the crystalline silica aerosol at a concentration of 15  $\text{mg m}^{-3}$ , 6 h per day for 5 consecutive days. The concentration of silica exposure employed in this study was higher than the minimum concentration (2  $\text{mg m}^{-3}$ , 6 h per day, for 5 consecutive days) reported to be required to result in slight pulmonary toxicity in the rats (Sellamuthu *et al.*, 2011). Forty rats exposed simultaneously to filtered air served as the controls. The control and silica-exposed rats (eight rats per group) were sacrificed following an ip injection of 100 mg sodium pentobarbital  $\text{kg}^{-1}$  body weight (Fort Dodge, IA, USA) at post-exposure time intervals of 1, 2, 4, 8 and 16 weeks following termination of the one week silica exposure. The lung samples from the control and silica-exposed rats were collected to determine pulmonary toxicity and the findings have been reported recently (Sellamuthu *et al.*, 2011). Simultaneously, RNA was isolated from the lung samples to determine global gene expression profile as described below.

### RNA Isolation

Total RNA was isolated from rat lung samples using RNeasy Fibrous Tissue Mini Kit (Qiagen Inc., Valencia, CA, USA). Briefly, 20–30 mg lung tissue, obtained from the unlavaged upper lobe of the right lungs of the control and silica-exposed rats, was homogenized in Buffer RLT containing  $\beta$ -mercaptoethanol and two 2.4 mm Zirconia Beads (BioSpec Products Inc., Bartlesville, OK, USA) using a Mini beadbeater-8 (BioSpec Products Inc.) for 20 s. After centrifugation at 10 000  $g$  for 3 min at room temperature, the supernatant containing RNA was isolated and applied to the RNeasy column and processed as directed in the RNeasy Fibrous Tissue Mini Kit protocol. The integrity of the RNA samples was determined using the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) and quantitated by UV spectrophotometry. Only RNA samples that exhibited an RNA integrity number (RIN) >8.0 were used in the gene expression analysis.

### Microarray Analysis of Global Gene Expression Profile

The RNA isolated from the rat lung samples was analyzed for global gene expression profiling using RatRef-12 V1.0 Expression BeadChip arrays (Illumina, Inc, San Diego, CA, USA). All microarray experiments were performed to comply with Minimal Information About a Microarray Experiment (MIAME) protocols. Biotin-labeled cRNA was generated from 375 ng total RNA samples each by employing the Illumina TotalPrep RNA Amplification Kit (Ambion, Inc, Austin, TX, USA). Chip hybridizations, washing, Cy3-streptavidin staining and scanning of the chips on the Beadstation 500 platform (Illumina Inc.) were performed following the protocols provided by the manufacturer.

Metric files from the bead scanner were checked to ensure that all samples fluoresced at comparable levels before samples were loaded into Beadstudio (Framework version 3.0.19.0) Gene Expression module version 3.0.14. Housekeeping, hybridization control, stringency and negative control genes were checked for proper chip detection. BeadArray

expression data were then exported with mean fluorescent intensity across like beads and bead variance estimates into flat files for subsequent analysis.

Illumina BeadArray expression data was analyzed in Bioconductor using the 'lumi' and 'limma' packages. Bioconductor is a project for the analysis and comprehension of genomic data and operates in R, a statistical computing environment (Ihaka and Gentleman, 1996). The 'lumi' Bioconductor package was specifically developed to process Illumina microarrays and covers data input, quality control, variance stabilization, normalization and gene annotation (Gentleman *et al.*, 2004). Normalized data were then analyzed using the 'limma' package in R. In short, limma fits a linear model for each gene, generates group means of expression and calculates *P*-values and log fold-changes which are converted to standard fold changes. The raw *P*-values were corrected for false discovery rate (FDR) using the Benjamini and Hochberg procedure (Benjamini and Hochberg, 1995). Only genes with FDR *P*-value <0.01 and a fold change in expression >1.5 compared with the corresponding time-matched controls were considered as significantly differentially expressed genes (SDEGs) and used as input for subsequent bioinformatics analysis.

### Quantitative Real-time Polymerase Chain Reaction Analysis

Ten genes exhibiting a significant (FDR *P* <0.01 and fold change >1.5) silica exposure-dependent overexpression in the rat lungs compared with the time-matched controls were selected for QRT-PCR analysis to confirm the microarray data. The nucleotide sequences of the primers used in the quantitative real-time polymerase chain reaction (QRT-PCR) analysis of the selected genes and the housekeeping genes, *β-actin*, are presented in Table 1. The PCR amplification, detection of the PCR amplified gene products, and their quantitations were performed with a 7900 HT fast real-time PCR machine and the SYBR Green PCR MasterMix (Applied Biosystems, Foster City, CA, USA). The specificity and integrity of the PCR products were determined by analyzing the melting curves of all PCR amplified gene products. The expression levels of the genes were normalized to that of the housekeeping gene and the fold changes in expression compared with the controls were calculated using the formula  $2^{[-(C_{t\text{target}} - C_{t\text{housekeeping gene}})]}$ .

### Bioinformatics Analysis of SDEGs

Bioinformatics analysis of the SDEGs was conducted using Ingenuity Pathway Analysis (IPA, Ingenuity Systems, [www.ingenuity.com](http://www.ingenuity.com)). IPA software is designed to map the biological relationship of the uploaded genes and classify them into categories of biological functions, molecular networks or canonical pathways according to published literature in the database. Fisher's exact test was conducted to calculate the *P*-value determining the significance of a particular biological function, molecular network and canonical pathway enriched by silica exposure in the rat lungs. The criteria required for a biological function, molecular network and canonical pathway to be considered significantly enriched in response to silica exposure and toxicity was that the category within a gene set was represented with a significance level for enrichment of *P* <0.05. The biological functions, molecular networks and canonical pathways significantly enriched (*P* <0.05) but represented by fewer than five genes were filtered out to identify the functions, networks, and pathways most robustly perturbed by silica exposure in the rat lungs.

## Statistical Analysis of the Data

Nonmicroarray data between the silica-exposed and corresponding time-matched control group of rats were compared using the one-way ANOVA test. *Post hoc* comparisons were made with Fisher's least significant difference (LSD) test. The level of statistical significance was set at  $P < 0.05$ .

## RESULTS

### Progression of Pulmonary Toxicity in the Silica-exposed Rats

A summary of our recently published findings on silica induced pulmonary toxicity in the rats employed in this study is presented in Table 2. The silica-induced pulmonary toxicity, in general, exhibited a steady progression during the post-exposure time intervals analyzed as evidenced from the various biochemical, histological and cellular toxicity parameters determined in the rats.

### Lung Gene Expression Profile

Microarray analysis of the global gene expression profile identified the genes whose expressions were significantly affected by silica exposure in the lungs of rats (Supporting Information, tables 1–5). The number of SDEGs identified in the lungs of the silica-exposed rats, compared with the corresponding time-matched control rats, exhibited a steady increase during the post-exposure time intervals analyzed (Fig. 1). The increase in the number of SDEGs in the silica-exposed rats exhibited a trend similar to the various pulmonary toxicity parameters observed during the post-exposure time intervals.

Results of QRT-PCR analysis further confirmed the microarray data. In agreement with the microarray results, all 10 representative genes selected for QRT-PCR analysis exhibited a significant ( $P < 0.05$ ) overexpression in the lungs of the silica-exposed rats (Fig. 2A and B). In general, the progressive increase in the magnitude of overexpression of most of the representative genes observed in the silica-exposed rat lungs during the post-exposure time intervals was confirmed by the results of QRT-PCR analysis.

### Bioinformatics Analysis of SDEGs

Bioinformatics analysis of the SDEGs obtained from the microarray analysis identified the various biological functions, canonical pathways and molecular networks that were significantly enriched in the rat lungs by inhalation exposure to silica. In agreement with the results of silica-induced pulmonary toxicity (Table 2) and the number of SDEGs (Fig. 1), a similar trend was noticed with respect to the number of IPA biological functions, molecular networks and canonical pathways, which were significantly enriched by silica exposure in the rat lungs (Fig. 3A–C). The top ranking biological functions significantly affected by silica exposure were inflammatory response, cell-to-cell signaling and interaction, cellular movement, inflammatory diseases, respiratory diseases and cancer (Fig. 4). The number of SDEGs belonging to each of these top ranking biological functions, as in the case of silica-induced pulmonary toxicity (Table 2), also exhibited a steady increase during the post-exposure time intervals analyzed (Fig. 4). The vast majority of the significantly enriched canonical pathways in the silica-exposed rat lungs were those involved in an inflammatory

response (Supporting Information, table 6). The time-course of enrichment of acute phase response and complement system in the silica-exposed rat lungs during the post-exposure time intervals are presented as representative canonical pathways enriched by silica exposure in the rats (Fig. 5A and B). The involvement of both these canonical pathways, as evidenced by their IPA *P*-values, in the pulmonary response of the silica-exposed rat lungs also exhibited a steady increase during the post-exposure time intervals analyzed. The number of molecular networks significantly enriched in the rat lungs in response to pulmonary exposure to silica (Fig. 3B) as well as the number of molecules (genes) belonging to each of the networks exhibited a steady increase during the post-exposure time intervals, as was noticed in the case of silica-induced pulmonary toxicity (Table 2). A highly ranked molecular network consisting of 28 genes differentially expressed in the silica-exposed lungs at the 16-week post-exposure time interval and belonging to cell-to-cell signaling and interaction, cellular movement, and inflammatory response with nuclear factor kappa B (NF $\kappa$ B) as the central molecule is presented as a representative network to demonstrate its involvement in the progression of silica-induced pulmonary toxicity (Fig. 6). There was a steady and post-silica exposure time-dependent increase in the number of SDEGs in the silica-exposed rat lungs interacting with one another and thus constituting the representative NF $\kappa$ B network, as was noticed in the case of silica-induced pulmonary toxicity in the rats. A selected list of SDEGs belonging to the various biological functions, pathways and networks that are significantly enriched and, therefore, are considered to be of importance in the silica-induced pulmonary toxicity is presented in Table 3 and the functional significance of their differential expression with respect to the progression of silica-induced pulmonary toxicity is discussed below.

## DISCUSSION

A comprehensive understanding of the molecular mechanisms underlying the initiation and progression of silica-induced pulmonary toxicity, which is critical in the potential prevention of diseases associated with silica exposure, is still lacking. The present study is part of an on-going research project aiming to identify the molecular targets and mechanisms underlying silica-induced pulmonary toxicity. Recently, we have reported developing a rat model for silica-induced pulmonary toxicity (Sellamuthu *et al.*, 2011). As summarized in Table 2, inhalation exposure of rats to silica (15 mg m<sup>-3</sup>, 6 h per day, 5 days) resulted in significant pulmonary toxicity as evidenced from elevations in bronchoalveolar lavage fluid (BALF) parameters of pulmonary toxicity [lactate dehydrogenase (LDH) activity, total protein and albumin levels], inflammation [number of alveolar macrophages (AMs) and polymorphonuclear leukocytes (PMNs) and concentrations of monocyte chemotactic protein-1 (MCP-1) and macrophage inflammatory protein-2 (MIP-2)] as well as histological changes in the lungs. The silica-induced pulmonary toxicity in these rats, in agreement with previous reports (Johnston *et al.*, 2000; Porter *et al.*, 2004), progressed steadily even after cessation of silica exposure. Presently, the lung samples obtained from these rats were analyzed by microarray to determine a global gene expression profile in order to identify the molecular targets as well as to elucidate the molecular mechanisms underlying the progression of silica-induced pulmonary toxicity.

The rat model for silica-induced pulmonary toxicity employed in this study is relevant to human silicosis. Epidemiologic studies have found that silicosis may develop and/or progress in workers even after occupational exposure to silica has ended (Kreiss and Zhen, 1996; Miller *et al.*, 1998). Analogous to the human situation, progression of silica-induced pulmonary toxicity for a prolonged period after cessation of silica exposure was observed in the rat model employed in this study (Table 2).

The results presented in this report justified the application of global gene expression profiling as a relevant approach to identify the molecular targets as well as to elucidate the molecular mechanisms underlying the progression of silica-induced pulmonary toxicity. As presented in Table 4, the various pulmonary toxicity parameters correlated well with the gene expression findings in the silica-exposed rats. In addition, results of the bioinformatics analysis of the SDEGs, in agreement with the findings of several previous studies, reaffirmed the ability of silica exposure to result in the induction of inflammation (Barbarin *et al.*, 2005), oxidative stress (Porter *et al.*, 2002), cancer (IARC, 1997) and respiratory diseases (Cooper *et al.*, 2002).

Generation of reactive oxygen species directly from silica particles (Vallyathan *et al.*, 1995) and/or indirectly by silica-induced cellular-mediated processes (Vallyathan *et al.*, 1992) has been found responsible, at least in part, for the silica-induced pulmonary toxicity. Significant overexpression of several oxidative stress-response genes, for example, *MT1A* (Chiaverini and De Ley, 2010), *HMOX1* (Joseph *et al.*, 2008), *LCN2* (Roudkenar *et al.*, 2007) and *ARG1* (Ogino *et al.*, 2011), noticed in the lung samples (Table 3) suggest possible induction of oxidative stress in the silica-exposed rat lungs. The *NOXO1* gene codes a protein that is an activator of the superoxide-generating gene NOX1 (Banfi *et al.*, 2003), and its significant overexpression in the silica-exposed rat lungs may, therefore, imply the generation of superoxide anion potentially capable of resulting in oxidative stress. In addition, the significant overexpression of *SOD2*, a gene responsible for dismutation of superoxide anion resulting in the generation of toxic hydrogen peroxide ( $H_2O_2$ ; Liochev and Fridovich, 2007), with no corresponding increase in the expression of  $H_2O_2$  detoxifying genes, *catalase* and *glutathione peroxidase/reductase* (Gaetani *et al.*, 1994), seen in the rat lungs should be expected to favor the excessive generation and tissue accumulation of reactive and toxic  $H_2O_2$ , contributing to oxidative stress and pulmonary toxicity in the silica-exposed rat lungs. This argument is further supported by the significant overexpression of *LPO*, an  $H_2O_2$ -responsive gene (Davies *et al.*, 2008), noticed in the silica-exposed rat lungs, especially during the late post-exposure time intervals (Table 3).

A central role for inflammation in the pulmonary effects associated with silica exposure has been established (Castranova, 2004). Significant increase in the number of AMs and PMNs and concentrations of the pro-inflammatory chemokines, MCP1 and MIP2, noticed in the lung samples used in this study (Table 2), suggested the induction of significant pulmonary inflammation in our rat model. The silica-induced pulmonary inflammation in the rats, similar to the trend exhibited by the various parameters of silica-induced pulmonary toxicity, exhibited a steady progression during the post-exposure time intervals analyzed (Table 2). Bioinformatics analysis of the SDEGs supported the induction and progression of pulmonary inflammation and toxicity noticed in the silica-exposed rats. Inflammatory

response, inflammatory diseases and cellular movement were three of the top ranking IPA biological functions identified as being significantly enriched by silica exposure in the rat lungs (Fig. 4). In addition, multiple canonical pathways and molecular networks involved in the induction of inflammation were significantly and progressively enriched during the silica post-exposure time intervals in the rats (Figs 5 and 6). Interestingly, the number of inflammation-related biological functions, pathways and networks that were significantly affected by silica exposure in the lungs also steadily increased (Figs 4–6) along with the progression of silica-induced pulmonary toxicity in the rats (Table 2), suggesting a possible relationship between silica-induced differential expression of genes involved in inflammation and the toxicity progression noticed in the rat lungs.

Gene expression profiling and bioinformatics analysis of the SDEGs also provided insights into the molecular mechanisms underlying the progression of silica-induced pulmonary inflammation and toxicity in the rats. Several inflammatory response genes that encode inflammatory cytokines/chemokines were significantly overexpressed in silica-exposed rat lungs and the magnitude of their overexpression steadily increased during the post-exposure time intervals along with the progression of pulmonary toxicity induced by silica in the rats (Table 3). Many of these pro-inflammatory cytokines/chemokines function as chemoattractants and recruit inflammatory cells, especially PMNs, into the lungs (Olson and Ley, 2002) in response to pulmonary damage and may, therefore, account, at least in part, for the significant increase in the number of PMNs detected in the lungs resulting in the induction and progression of pulmonary inflammation and toxicity as noticed in the silica-exposed rats (Sellamuthu *et al.*, 2011). In addition to the genes encoding inflammatory cytokines/chemokines, significant overexpression of several other genes that are known to play prominent roles in the induction of inflammation such as *S100A8* (Ryckman *et al.*, 2003), *RETNLA* (Holcomb *et al.*, 2000), *TREM1* and *TREM2* (Ford and McVicar, 2009), *LCN2* (Zhang *et al.*, 2008), *CHI3L1* (Eurich *et al.*, 2009), *SPP1* (Sabo-Attwood *et al.*, 2011), and several members of the complement system (Li *et al.*, 2007) and acute phase response (Whicher *et al.*, 1999) were found in the silica-exposed rat lungs (Table 3). It is noteworthy that overexpression of all these inflammatory response genes steadily increased along with the progression of silica-induced pulmonary inflammation and toxicity in the rats during the post-exposure time intervals analyzed, further supporting their involvement in the progression of pulmonary inflammation and toxicity in the silica-exposed rats.

Lipoxins play an important role in the resolution of pulmonary inflammation (Chan and Moore, 2010), and the involvement of lipoxins, if any, in silica-induced pulmonary inflammation has not been investigated to date. Even though the pulmonary level of lipoxins was not measured in the silica-exposed rats, our gene expression data provided indirect evidence for the involvement of lipoxins in silica-induced pulmonary inflammation. Lipoxins are products of arachidonic acid metabolism catalyzed by 15-lipoxygenase (Alox15; Kronke *et al.*, 2009). An anti-inflammatory role has been attributed to lipoxins mainly because of their ability to inhibit chemotaxis, adhere and transmigrate neutrophils and antagonize the pro-inflammatory effects of leukotrienes (Colgan *et al.*, 1993; Scalia *et al.*, 1997; Godson and Brady, 2000). *Alox-15* expression was significantly lower in the lungs of the silica-exposed rats compared with the time-matched controls (Table 3). Therefore, it

is reasonable to assume that, in addition to the significant overexpression of the multiple pro-inflammatory genes, the significant down-regulation of *Alox-15* gene expression might have contributed to the establishment of unresolved pulmonary inflammation noticed in the silica-exposed rats.

Pulmonary fibrosis is a major component of silicosis (Ng and Chan, 1991), the most serious health outcome of occupational exposure to silica. Even though significant histological pre-fibrotic changes, including type II pneumocyte hyperplasia, occurred in the rat lungs at 16 weeks following cessation of silica exposure (Table 2), pulmonary fibrosis had not developed at this stage. However, after a prolonged post-exposure time interval of 32 weeks, positive trichrome staining indicative of pulmonary fibrosis was observed in the silica-exposed rat lungs (unpublished data). Bioinformatics analysis of the gene expression data, on the other hand, identified significant differential expression of several genes involved in tissue remodeling and fibrosis in the lungs of the silica-exposed rats as early as one week after cessation of silica exposure (Table 3). Matrix metalloproteinases (MMPs) are a family of proteins participating in many normal biological processes as well as in pathological processes, including fibrotic lung diseases (Nagase and Woessner, 1999). Of the *MMPs* that were significantly overexpressed in the silica-exposed rat lungs, *MMP12* overexpression was most significant (Table 3 and Fig. 2). A definite role for *MMP12* in the induction of pulmonary fibrosis has been demonstrated previously in mice carrying a targeted deletion of the *MMP12* gene (Matute-Bello *et al.*, 2007). Osteopontin, one of the key components of extracellular matrix, mediates the migration, adhesion and proliferation of fibroblasts culminating in pulmonary fibrosis (Takahashi *et al.*, 2001). The profibrotic gene *SPPI*, which codes osteopontin protein, was significantly overexpressed in the silica-exposed rat lungs with increased overexpression at late post-exposure time intervals of 8 and 16 weeks (Table 3). The *ARG1* gene which was significantly and progressively overexpressed in the silica-exposed lung samples (Table 3) has been found to be associated with bleomycin-induced pulmonary fibrosis in mice (Endo *et al.*, 2003). The significant overexpression of the profibrotic chemokines such as *CCl2* (Mercer *et al.*, 2009) and *CCl7* (Moore and Hogaboam, 2008) observed in the silica-exposed rat lungs (Table 3) may indicate their involvement in silica-induced pulmonary fibrosis in the rats. This view is further supported by the significant overexpression of these chemokines in tuberculosis, a human fibrotic disease (Nau *et al.*, 1997). The involvement of *RETNLA* in the induction of pulmonary fibrosis by promoting the differentiation of myoblasts that mediate collagen deposition has been suggested (Liu *et al.*, 2004). The *RETNLA* gene was highly overexpressed in the silica-exposed rat lungs (Table 3). Since a definite relationship is known to exist between unresolved pulmonary inflammation and fibrosis (Reynolds, 2005), it is reasonable to assume that the significant overexpression of the several pro-inflammatory genes presented in Table 3 and described above and the resulting unresolved pulmonary inflammation observed in the rat lungs might be of significance in the context of silica-induced pulmonary fibrosis. The magnitude of overexpression of all these genes that are known to be involved in tissue remodeling and fibrosis steadily increased in parallel with the progression of silica-induced pulmonary toxicity in the rats, suggesting their potential contribution to silica-induced pulmonary fibrosis and toxicity.

The findings of the present transcriptomics study also provided novel insights into the mechanisms potentially underlying the progression of silica-induced pulmonary toxicity related to upper airway diseases. Significant overexpression of several members of the solute carrier (SLC) family of genes was noticed in the lungs of the silica-exposed rats (Fig. 2 and Table 3). The significant overexpression of the *SLC* genes exhibited a steady increase during the post-exposure time intervals (Table 3) in parallel with the progression of pulmonary toxicity noticed in the silica-exposed rats (Table 2), suggesting their potential involvement in the progression of silica-induced pulmonary toxicity. The SLC gene that was most significantly overexpressed in the lungs of the silica-exposed rats over time was *SLC26A4*, and several lines of evidence suggest the potential role of this gene in the initiation and progression of silica-induced pulmonary toxicity. The steady increase in the overexpression of *SLC26A4* gene transcript (Table 3 and Fig. 2) corresponded with the progression of pulmonary toxicity noticed in the rats (Table 2). The *SLC26A4* gene codes the protein, pendrin, which is responsible for excessive mucus production by airway epithelial cells (Nakao *et al.*, 2008), and a relationship exists between excessive mucus production by airway epithelial cells and morbidity and mortality from certain respiratory diseases (Rogers, 2004; Rose and Voynow, 2006). The steady increase in the overexpression of the *SLC26A4* gene noticed in the silica-exposed rats may also account, at least in part, for the progression of pulmonary inflammation noticed in our rat model. It has been reported previously that forced overexpression of *SLC26A4* gene, by yet to be identified mechanisms, results in the activation of the *CXC11* and *CXC12* chemoattractants and facilitates the infiltration of neutrophils into lungs, resulting in the induction of pulmonary inflammation (Nakao *et al.*, 2008). In this regard, it is important to notice that both *CXC11* and *CXC12* genes were significantly and progressively overexpressed (Table 3) and a significant increase in the number of infiltrating PMNs and induction of inflammation (Table 2) was noticed in our rat model. Collectively, the findings of this study and those reported previously (Nakao *et al.*, 2008) may suggest the involvement of the *SLC26A4* gene in the progression of silica-induced pulmonary inflammation and toxicity in the rats. The availability of the *SLC26A4* transgenic mouse (Lu *et al.*, 2011) should facilitate future investigations to further understand and confirm the role of this gene in silica-induced pulmonary toxicity.

In summary, the data presented in this report provided insights into the molecular targets and mechanisms underlying the progression of silica-induced pulmonary toxicity in a rat model that is relevant to human silicosis. In addition to confirming the central role played by unresolved inflammation in the pulmonary effects of silica exposure, our transcriptomics data provided insights into the molecular mechanisms, including novel ones, potentially underlying the pulmonary effects of silica exposure. It is anticipated that the data obtained in this investigation may be employed in the future to develop strategies to intervene and/or to prevent the adverse health effects associated with occupational exposure to silica.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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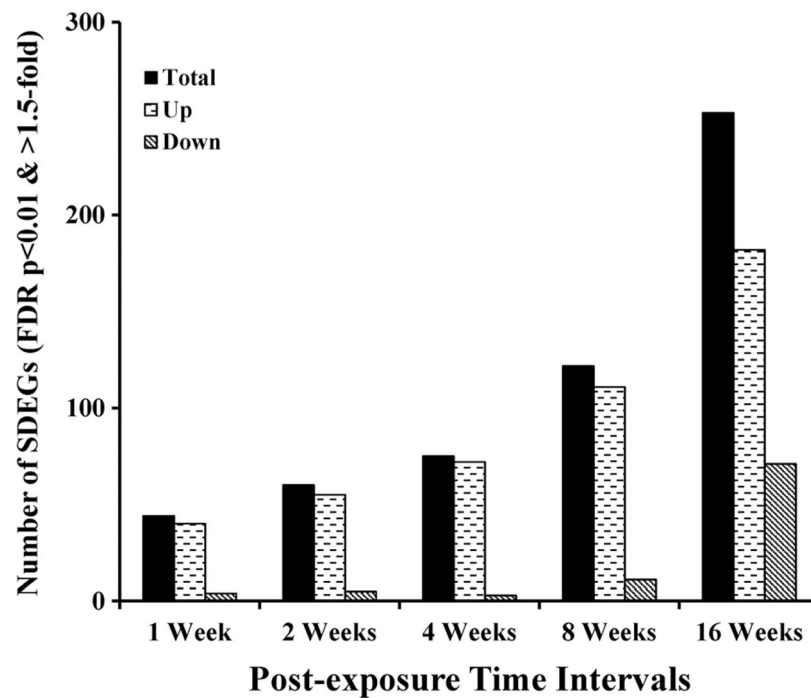
## References

- Amin RP, Vickers AE, Sistare F, Thompson KL, Roman RJ, Lawton M, Kramer J, Hamadeh HK, Collins J, Grissom S, Bennett L, Tucker CJ, Wild S, Kind C, Oreffo V, Davis JW 2nd, Curtiss S, Naciff JM, Cunningham M, Tennant R, Stevens J, Car B, Bertram TA, Afshari CA. Identification of putative gene based markers of renal toxicity. *Environ Health Perspect.* 2004; 112:465–479. [PubMed: 15033597]
- Banfi B, Clark RA, Steger K, Krause KH. Two novel proteins activate superoxide generation by the NADPH oxidase NOX1. *J Biol Chem.* 2003; 278:3510–3513. [PubMed: 12473664]
- Barbarin V, Nihoul A, Misson P, Arras M, Delos M, Leclercq I, Lison D, Huaux F. The role of pro- and anti-inflammatory responses in silica-induced lung fibrosis. *Respir Res.* 2005; 6:112. [PubMed: 16212659]
- Benjamini Y, Hochberg Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J R Stat Soc Ser.* 1995; 57:289–300.
- Castranova V. Signaling pathways controlling the production of inflammatory mediators in response to crystalline silica exposure: role of reactive oxygen/nitrogen species. *Free Radic Biol Med.* 2004; 37:916–925. [PubMed: 15336307]
- Chan MM, Moore AR. Resolution of inflammation in murine auto-immune arthritis is disrupted by cyclooxygenase-2 inhibition and restored by prostaglandin E2-mediated lipoxin A4 production. *J Immunol.* 2010; 184:6418–6426. [PubMed: 20435922]
- Chiaverini N, De Ley M. Protective effect of metallothionein on oxidative stress-induced DNA damage. *Free Radic Res.* 2010; 44:605–613. [PubMed: 20380594]
- Colgan SP, Serhan CN, Parkos CA, Delp-Archer C, Madara JL. Lipoxin A4 modulates transmigration of human neutrophils across intestinal epithelial monolayers. *J Clin Invest.* 1993; 92:75–82. [PubMed: 8326019]
- Cooper GS, Miller FW, Germolec DR. Occupational exposures and autoimmune diseases. *Int Immunopharmacol.* 2002; 2:303–313. [PubMed: 11811933]
- Davies MJ, Hawkins CL, Pattison DI, Rees MD. Mammalian heme peroxidases: from molecular mechanisms to health implications. *Anti-oxid Redox Signal.* 2008; 10:1199–1234.
- Endo M, Oyadomari S, Terasaki Y, Takeya M, Suga M, Mori M, Gotoh T. Induction of arginase I and II in bleomycin-induced fibrosis of mouse lung. *Am J Physiol Lung Cell Mol Physiol.* 2003; 285:L313–321. [PubMed: 12679322]
- Eurich K, Segawa M, Toei-Shimizu S, Mizoguchi E. Potential role of chitinase 3-like-1 in inflammation-associated carcinogenic changes of epithelial cells. *World J Gastroenterol.* 2009; 15:5249–5259. [PubMed: 19908331]
- Fielden MR, Nie A, McMillian M, Elangbam CS, Trela BA, Yang Y, Dunn RT 2nd, Dragan Y, Fransson-Stehen R, Bogdanffy M, Adams SP, Foster WR, Chen SJ, Rossi P, Kasper P, Jacobson-Kram D, Tatsuoka KS, Wier PJ, Gollub J, Halbert DN, Roter A, Young JK, Sina JF, Marlowe J, Martus HJ, Aubrecht J, Olaharski AJ, Roome N, Nioi P, Pardo I, Snyder R, Perry R, Lord P, Mattes W, Car BD. Interlaboratory evaluation of genomic signatures for predicting carcinogenicity in the rat. *Toxicol Sci.* 2008; 103:28–34. [PubMed: 18281259]
- Ford JW, McVicar DW. TREM and TREM-like receptors in inflammation and disease. *Curr Opin Immunol.* 2009; 21:38–46. [PubMed: 19230638]
- Foster WR, Chen SJ, He A, Truong A, Bhaskaran V, Nelson DM, Dambach DM, Lehman-McKeeman LD, Car BD. A retrospective analysis of toxicogenomics in the safety assessment of drug candidates. *Toxicol Pathol.* 2007; 35:621–635. [PubMed: 17654404]
- Gaetani GF, Kirkman HN, Mangerini R, Ferraris AM. Importance of catalase in the disposal of hydrogen peroxide within human erythrocytes. *Blood.* 1994; 84:325–330. [PubMed: 8018928]

- Gentleman RC, Carey VJ, Bates DM, Bolstad B, Dettling M, Dudoit S, Ellis B, Gautier L, Ge Y, Gentry J, Hornik K, Hothorn T, Huber W, Iacus S, Irizarry R, Leisch F, Li C, Maechler M, Rossini AJ, Sawitzki G, Smith C, Smyth G, Tierney L, Yang JY, Zhang J. Bioconductor: open software development for computational biology and bioinformatics. *Genome Biol.* 2004; 5:R80. [PubMed: 15461798]
- Godson C, Brady HR. Lipoxins: novel anti-inflammatory therapeutics? *Curr Opin Investig Drugs.* 2000; 1:380–385.
- Hamadeh HK, Knight BL, Haugen AC, Sieber S, Amin RP, Bushel PR, Stoll R, Blanchard K, Jayadev S, Tennant RW, Cunningham ML, Afshari CA, Paules RS. Methapyrilene toxicity: anchorage of pathologic observations to gene expression alterations. *Toxicol Pathol.* 2002; 30:470–482. [PubMed: 12187938]
- Holcomb IN, Kabakoff RC, Chan B, Baker TW, Gurney A, Henzel W, Nelson C, Lowman HB, Wright BD, Skelton NJ, Frantz GD, Tumas DB, Peale FV Jr, Shelton DL, Hebert CC. FIZZ1, a novel cysteine-rich secreted protein associated with pulmonary inflammation, defines a new gene family. *EMBO J.* 2000; 19:4046–4055. [PubMed: 10921885]
- IARC. Monograph on the Evaluation of Carcinogenic Risk to Human. Vol. 68. International Agency for Research on Cancer; 1997. p. 1-475.
- Ihaka R, Gentleman R. R: a language for data analysis and graphics. *J Comput Graphic Stat.* 1996; 5:299–314.
- Johnston CJ, Driscoll KE, Finkelstein JN, Baggs R, O'Reilly MA, Carter J, Gelein R, Oberdorster G. Pulmonary chemokine and mutagenic responses in rats after subchronic inhalation of amorphous and crystalline silica. *Toxicol Sci.* 2000; 56:405–413. [PubMed: 10911000]
- Joseph P, He Q, Umbright C. Heme-oxygenase 1 gene expression is a marker for hexavalent chromium-induced stress and toxicity in human dermal fibroblasts. *Toxicol Sci.* 2008; 103:325–334. [PubMed: 18332044]
- Kreiss K, Zhen B. Risk of silicosis in a Colorado mining community. *Am J Ind Med.* 1996; 30:529–539. [PubMed: 8909602]
- Kronke G, Katzenbeisser J, Uderhardt S, Zaiss MM, Scholtysek C, Schabbauer G, Zarbock A, Koenders MI, Axmann R, Zwerina J, Baenckler HW, van den Berg W, Voll RE, Kuhn H, Joosten LA, Schett G. 12/15-lipoxygenase counteracts inflammation and tissue damage in arthritis. *J Immunol.* 2009; 183:3383–3389. [PubMed: 19675173]
- Li M, Peake PW, Charlesworth JA, Tracey DJ, Moalem-Taylor G. Complement activation contributes to leukocyte recruitment and neuropathic pain following peripheral nerve injury in rats. *Eur J Neurosci.* 2007; 26:3486–3500. [PubMed: 18052971]
- Linch KD, Miller WE, Althouse RB, Groce DW, Hale JM. Surveillance of respirable crystalline silica dust using OSHA compliance data (1979–1995). *Am J Ind Med.* 1998; 34:547–558. [PubMed: 9816412]
- Liochev SI, Fridovich I. The effects of superoxide dismutase on H<sub>2</sub>O<sub>2</sub> formation. *Free Radic Biol Med.* 2007; 42:1465–1469. [PubMed: 17448892]
- Liu T, Jin H, Ullenbruch M, Hu B, Hashimoto N, Moore B, McKenzie A, Lukacs NW, Phan SH. Regulation of found in inflammatory zone 1 expression in bleomycin-induced lung fibrosis: role of IL-4/IL-13 and mediation via STAT-6. *J Immunol.* 2004; 173:3425–3431. [PubMed: 15322207]
- Lu YC, Wu CC, Shen WS, Yang TH, Yeh TH, Chen PJ, Yu IS, Lin SW, Wong JM, Chang Q, Lin X, Hsu CJ. Establishment of a knock-in mouse model with the SLC26A4 c. 919-2A>G mutation and characterization of its pathology. *PLoS One.* 2011; 6:e22150. [PubMed: 21811566]
- Matute-Bello G, Wurfel MM, Lee JS, Park DR, Frevert CW, Madtes DK, Shapiro SD, Martin TR. Essential role of MMP-12 in Fas-induced lung fibrosis. *Am J Respir Cell Mol Biol.* 2007; 37:210–221. [PubMed: 17446527]
- Mercer PF, Johns RH, Scotton CJ, Krupiczkoj MA, Konigshoff M, Howell DC, McAnulty RJ, Das A, Thorley AJ, Tetley TD, Eickelberg O, Chambers RC. Pulmonary epithelium is a prominent source of proteinase-activated receptor-1-inducible CCL2 in pulmonary fibrosis. *Am J Respir Crit Care Med.* 2009; 179:414–425. [PubMed: 19060230]

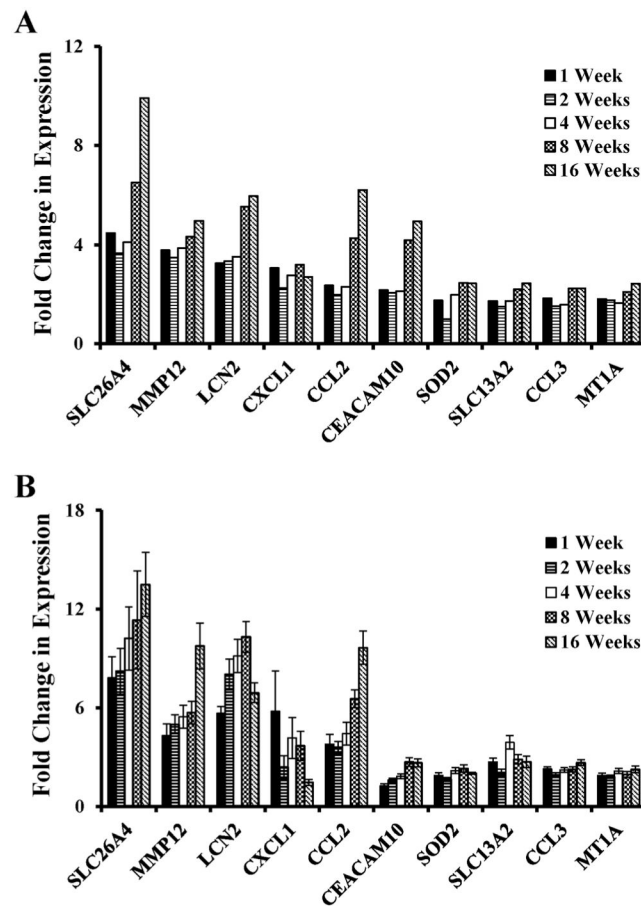
- Miller BG, Hagen S, Love RG, Soutar CA, Cowie HA, Kidd MW, Robertson A. Risks of silicosis in coalworkers exposed to unusual concentrations of respirable quartz. *Occup Environ Med*. 1998; 55:52–58. [PubMed: 9536164]
- Moore BB, Hogaboam CM. Murine models of pulmonary fibrosis. *Am J Physiol Lung Cell Mol Physiol*. 2008; 294:L152–160. [PubMed: 17993587]
- Nagase H, Woessner JF Jr. Matrix metalloproteinases. *J Biol Chem*. 1999; 274:21491–21494. [PubMed: 10419448]
- Nakao I, Kanaji S, Ohta S, Matsushita H, Arima K, Yuyama N, Yamaya M, Nakayama K, Kubo H, Watanabe M, Sagara H, Sugiyama K, Tanaka H, Toda S, Hayashi H, Inoue H, Hoshino T, Shiraki A, Inoue M, Suzuki K, Aizawa H, Okinami S, Nagai H, Hasegawa M, Fukuda T, Green ED, Izuhara K. Identification of pendrin as a common mediator for mucus production in bronchial asthma and chronic obstructive pulmonary disease. *J Immunol*. 2008; 180:6262–6269. [PubMed: 18424749]
- Nau GJ, Guilfoile P, Chupp GL, Berman JS, Kim SJ, Kornfeld H, Young RA. A chemoattractant cytokine associated with granulomas in tuberculosis and silicosis. *Proc Natl Acad Sci USA*. 1997; 94:6414–6419. [PubMed: 9177232]
- Ng TP, Chan SL. Factors associated with massive fibrosis in silicosis. *Thorax*. 1991; 46:229–232. [PubMed: 2038729]
- Ogino K, Takahashi N, Takigawa T, Obase Y, Wang DH. Association of serum arginase I with oxidative stress in a healthy population. *Free Radic Res*. 2011; 45:147–155. [PubMed: 20942574]
- Olson TS, Ley K. Chemokines and chemokine receptors in leukocyte trafficking. *Am J Physiol Regul Integr Comp Physiol*. 2002; 283:R7–28. [PubMed: 12069927]
- Porter DW, Barger M, Robinson VA, Leonard SS, Landsittel D, Castranova V. Comparison of low doses of aged and freshly fractured silica on pulmonary inflammation and damage in the rat. *Toxicology*. 2002; 175:63–71. [PubMed: 12049836]
- Porter DW, Hubbs AF, Mercer R, Robinson VA, Ramsey D, McLaurin J, Khan A, Battelli L, Brumbaugh K, Teass A, Castranova V. Progression of lung inflammation and damage in rats after cessation of silica inhalation. *Toxicol Sci*. 2004; 79:370–380. [PubMed: 15056817]
- Reynolds HY. Lung inflammation and fibrosis: an alveolar macrophage-centered perspective from the 1970s to 1980s. *Am J Respir Crit Care Med*. 2005; 171:98–102. [PubMed: 15557133]
- Rogers DF. Airway mucus hypersecretion in asthma: an undervalued pathology? *Curr Opin Pharmacol*. 2004; 4:241–250. [PubMed: 15140415]
- Rose MC, Voynow JA. Respiratory tract mucin genes and mucin glycoproteins in health and disease. *Physiol Rev*. 2006; 86:245–278. [PubMed: 16371599]
- Roudkenar MH, Kuwahara Y, Baba T, Roushandeh AM, Ebishima S, Abe S, Ohkubo Y, Fukumoto M. Oxidative stress induced lipocalin 2 gene expression: addressing its expression under the harmful conditions. *J Radiat Res (Tokyo)*. 2007; 48:39–44. [PubMed: 17229997]
- Ryckman C, Vandal K, Rouleau P, Talbot M, Tessier PA. Proinflammatory activities of S100: proteins S100A8, S100A9, and S100A8/A9 induce neutrophil chemotaxis and adhesion. *J Immunol*. 2003; 170:3233–3242. [PubMed: 12626582]
- Sabo-Attwood T, Ramos-Nino ME, Eugenia-Ariza M, Macpherson MB, Butnor KJ, Vacek PC, McGee SP, Clark JC, Steele C, Mossman BT. Osteopontin modulates inflammation, mucin production, and gene expression signatures after inhalation of asbestos in a murine model of fibrosis. *Am J Pathol*. 2011; 178:1975–1985. [PubMed: 21514415]
- Scalia R, Gefen J, Petasis NA, Serhan CN, Lefer AM. Lipoxin A4 stable analogs inhibit leukocyte rolling and adherence in the rat mesenteric microvasculature: role of P-selectin. *Proc Natl Acad Sci USA*. 1997; 94:9967–9972. [PubMed: 9275235]
- Sellamuthu R, Umbright C, Roberts JR, Chapman R, Young SH, Richardson D, Leonard H, McKinney W, Chen B, Frazer D, Li S, Kashon M, Joseph P. Blood gene expression profiling detects silica exposure and toxicity. *Toxicol Sci*. 2011; 122:253–264. [PubMed: 21602193]
- Takahashi F, Takahashi K, Okazaki T, Maeda K, Ienaga H, Maeda M, Kon S, Uede T, Fukuchi Y. Role of osteopontin in the pathogenesis of bleomycin-induced pulmonary fibrosis. *Am J Respir Cell Mol Biol*. 2001; 24:264–271. [PubMed: 11245625]

- Umbright C, Sellamuthu R, Li S, Kashon M, Luster M, Joseph P. Blood gene expression markers to detect and distinguish target organ toxicity. *Mol Cell Biochem.* 2010; 335:223–234. [PubMed: 19784758]
- Vallyathan V, Castranova V, Pack D, Leonard S, Shumaker J, Hubbs AF, Shoemaker DA, Ramsey DM, Pretty JR, McLaurin JL, Khan A, Teass A. Freshly fractured quartz inhalation leads to enhanced lung injury and inflammation. Potential role of free radicals. *Am J Respir Crit Care Med.* 1995; 152:1003–1009. [PubMed: 7663775]
- Vallyathan V, Mega JF, Shi X, Dalal NS. Enhanced generation of free radicals from phagocytes induced by mineral dusts. *Am J Respir Cell Mol Biol.* 1992; 6:404–413. [PubMed: 1312851]
- Waring JF, Jolly RA, Ciurlionis R, Lum PY, Praestgaard JT, Morfitt DC, Buratto B, Roberts C, Schadt E, Ulrich RG. Clustering of hepatotoxins based on mechanism of toxicity using gene expression profiles. *Toxicol Appl Pharmacol.* 2001; 175:28–42. [PubMed: 11509024]
- Whicher J, Biasucci L, Rifai N. Inflammation, the acute phase response and atherosclerosis. *Clin Chem Lab Med.* 1999; 37:495–503. [PubMed: 10418738]
- Zhang J, Wu Y, Zhang Y, Leroith D, Bernlohr DA, Chen X. The role of lipocalin 2 in the regulation of inflammation in adipocytes and macrophages. *Mol Endocrinol.* 2008; 22:1416–1426. [PubMed: 18292240]



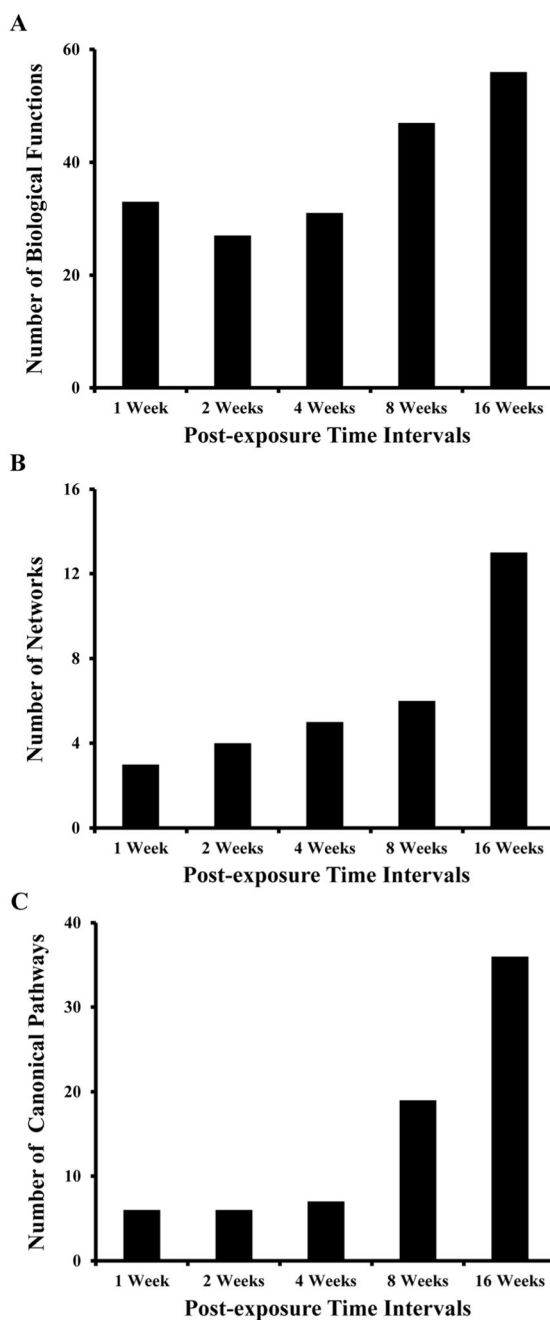
**Figure 1. Differential gene expression profile in the lungs of silica exposed rats**

Rats were exposed to silica (15 mg/m<sup>3</sup>, 6 hours/day, 5 days) and the number of significantly differentially expressed genes (SDEGs) ( $> 1.5$ -fold change and  $< 0.01$  FDR  $p$  value) was determined by microarray analysis. The number of genes differentially expressed (total), overexpressed (up) and under expressed (down) in the silica exposed rat lungs compared with the corresponding time-matched controls are presented for the post-exposure time intervals presented on the X-axis. Data represents the mean of eight silica exposed rats compared with four corresponding time-matched control rats per time point.



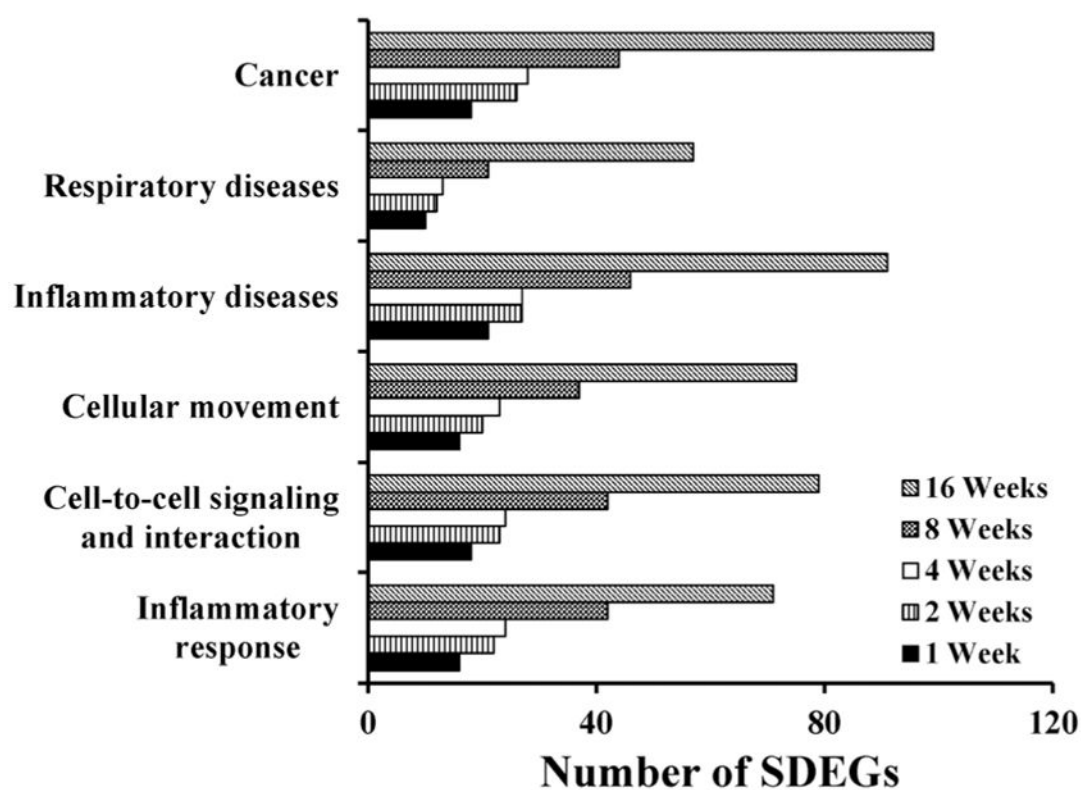
**Figure 2. Validation of microarray results by QRT-PCR**

A set of 10 genes which were significantly differentially expressed in the silica exposed rat lungs as evidenced from the microarray data presented in Figure 2A was analyzed by QRT-PCR as described in the Materials and methods section and the results are presented in Figure 2B. Data presented is the mean (A) or mean + S.E. (B) of fold-change in gene expression in the silica exposed rat lungs (n = 8) compared with the corresponding time-matched control rat lungs (n = 4). All gene expression changes were significant (FDR p < 0.01 in A and p < 0.05 in B) compared with the corresponding time matched controls.



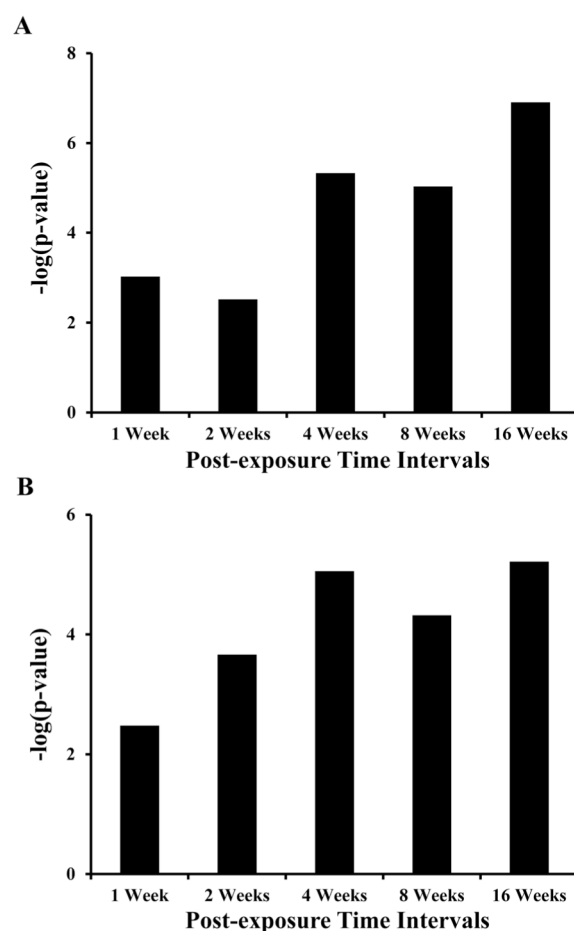
**Figure 3. Enrichment of biological functions, molecular networks, and canonical pathways in silica exposed rat lungs**

Bioinformatics analysis of the significantly differentially expressed genes in the silica exposed rat lungs was done using IPA software. The number of biological functions (A), molecular networks (B), and canonical pathways (C) significantly enriched in the silica exposed rat lungs ( $p < 0.05$ ) compared with the corresponding time matched controls are presented at each of the post-exposure time intervals presented on the X-axis. Data represents the group mean of eight silica exposed and four time-matched control rats per time point.



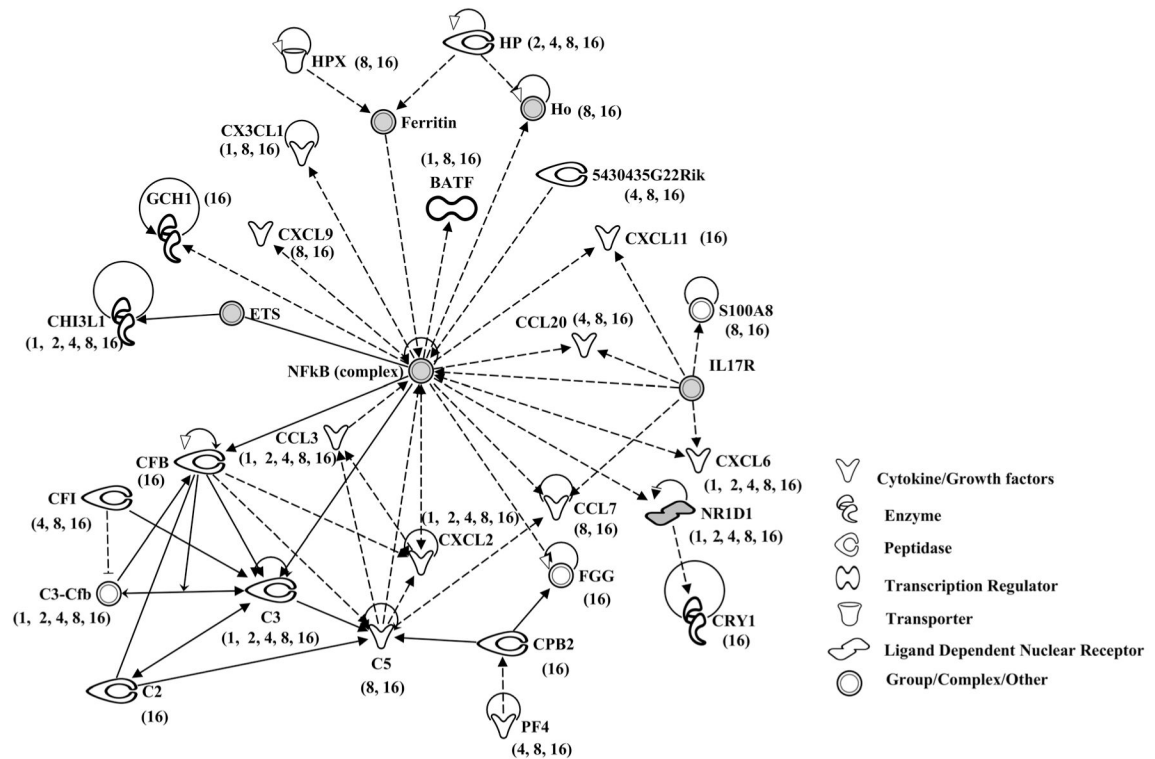
**Figure 4. Progression of enrichment of top ranking IPA biological functions in silica exposed rat lungs**

The number of significantly differentially expressed genes (SDEGs) in the silica exposed rat lungs belonging to the six top ranking IPA biological functions at each of the post-exposure time interval is presented. Data represents the group mean of eight silica exposed and four time-matched control rats per time point.



**Figure 5. Enrichment of complement system and acute phase response signaling canonical pathways in silica exposed rat lungs**

The complement system (A) and acute phase response signaling (B) are presented as representative IPA canonical pathways enriched in the silica exposed rat lungs. A complete listing of the IPA canonical pathways enriched in rat lungs in response to silica exposure is presented in Supporting information, table 6. Data represents the group mean of eight silica exposed and four time-matched control rats per time point.



**Figure 6. Enrichment of the NFκB molecular network in silica exposed rat lungs**

The NFκB molecular network was constructed using the genes that were significantly differentially expressed in the lungs of the silica exposed rats and belonging to the IPA function categories of cell-cell-signaling and interaction, cellular movement, and inflammatory response. The numbers presented in the parenthesis adjacent to the gene symbols are the silica post-exposure time interval in weeks at which the gene represented was significantly differentially expressed in the silica exposed rat lungs compared with the corresponding time-matched control rats. Data represents the group mean of eight silica exposed and four time-matched control rats per time point. *NR1D1* expression was significantly reduced while all other genes were significantly overexpressed in the silica exposed rat lungs.

**Table 1**

Nucleotide sequences of primers of genes selected for QRT-PCR analysis

Gene symbol <sup>a</sup>	Forward primer	Reverse primer
<i>SLC26A4</i>	GGGCAACCAAGAACGGGATTATAAG	TCTGGCTCTTCGACATCTTCATCAG
<i>MMP12</i>	GAGGTACGATGTGAGGCAGGAACTC	TTGGCCTGATTCTGGGAAGTG
<i>LCN2</i>	CAAGTCTCTGGGCCTCAAGGATAAC	CACCGTCTGTTCAGTTGTCAATGC
<i>CXCL1</i>	GGCAGGGATTCACTTCAAGAACATC	AGTGTGGCTATGACTTCGGTTTGG
<i>CCL2</i>	TGTCTCAGCCAGATGCAGTTAATGC	CAGCCGACTCATTGGGATCATC
<i>CEACAM10</i>	GGTACAAGGGAACCACTCCGAATC	AGGTCCTGTTTGATGCACATTATCG
<i>SOD2</i>	AGAAGGACACCCTTTCCTGACAAG	TGCACAGCTCTACACCCAAATGC
<i>SLC13A2</i>	CCAACGCCATTGTATTCTCCTTTG	TCCGATGATATTGAGCAGGAATCC
<i>CCL3</i>	CATTCTGCCCACCTGCAAATCTC	TTCAAGTGAAGAGTCCCTGGATGTG
<i>MT1A</i>	GCTGTGCCTGAAGTGACGAACAG	GGGTGGAGGTGTACGGCAAGAC
<i>β-Actin</i>	AGGGAAATCGTGCGTGACATTAAAG	GATGCGGCAGTGGCCATCTC

<sup>a</sup>The full nomenclature of the genes can be found in Table 3 and/or Supporting Information, table 5.

Summary of the pulmonary toxicity evaluation findings of crystalline silica exposed rats (adapted from Sellamuthu *et al.*, 2011)

Table 2

Toxicity parameter	Fold change compared to time matched control				
	1 Week	2 Weeks	4 Weeks	8 Weeks	16 Weeks
BALF LDH <sup>a</sup>	1.41	1.18	1.57	1.98	2.44
BALF Albumin <sup>b</sup>	1.45	1.11	1.45	1.70	1.58
BALF Protein <sup>b</sup>	1.08	0.96	1.31	1.25	1.27
BALF AM <sup>c</sup>	1.27	1.39	1.46	1.52	1.61
BALF PMN <sup>c</sup>	1.92	1.20	1.69	2.81	7.68
BALF MCP1 <sup>d</sup>	0.68	1.28	1.37	2.79	5.87
BALF MIP2 <sup>d</sup>	1.27	1.05	1.45	1.74	1.32
Histopathology <sup>e</sup>	None	None	None	Mild	Moderate

<sup>a</sup> Activity (U l<sup>-1</sup> BALF)

<sup>b</sup> concentration (mg l<sup>-1</sup> BALF)

<sup>c</sup> number (×10<sup>6</sup>)

<sup>d</sup> concentration (pg ml<sup>-1</sup> BALF); and

<sup>e</sup> none – no significant histological change, mild – accumulation of AM and infiltration of PMN, and moderate – accumulation of AM, infiltration of PMN, and type II pneumocyte hyperplasia.

Fold change in expression of a selected list of significantly differentially expressed genes in the lungs of silica exposed rats

**Table 3**

Gene	Fold change in expression (post-exposure time intervals)				
	1 Week	2 Weeks	4 Weeks	8 Weeks	16 Weeks
<b>Antioxidants and oxidative stress</b>					
<i>Superoxide dismutase 2 (SOD2)</i>	1.76*	1.85*	1.98*	2.47*	2.44*
<i>Heme oxygenase 1 (HMOX1)</i>	1.40*	1.39*	1.46*	1.58*	2.05*
<i>Metallothionein 1a (MT1A)</i>	1.81*	1.77*	1.65*	2.11*	2.44*
<i>NADPH oxidase organizer 1 (NOXO1)</i>	2.54*	2.07*	2.50*	3.24*	3.16*
<i>Lipocalin 2 (LCN2)</i>	3.25*	3.34*	3.58*	5.53*	5.96*
<i>Arginase 1 (ARG1)</i>	1.59*	1.29*	1.38*	1.93*	2.28*
<i>Lactoperoxidase (LPO)</i>	1.09*	1.02	1.03	1.23*	2.09*
<b>Cancer</b>					
<i>Lipocalin 2 (LCN2)</i>	3.25*	3.34*	3.54*	5.53*	5.96*
<i>Chitinase 3-like 1 (CHI3L1)</i>	1.89*	2.15*	2.17*	2.75*	3.34*
<i>Secreted phosphoprotein 1 (SPP1)</i>	1.34	1.01	1.12	1.85*	6.27*
<b>Inflammation</b>					
<i>Chemokine (C-C motif) ligand 2 (CCl2)</i>	2.36*	1.99*	2.30*	4.28*	6.22*
<i>Chemokine (C-C motif) ligand 3 (CCl3)</i>	1.84*	1.53*	1.58*	2.24*	2.25*
<i>Chemokine (C-C motif) ligand 4 (CCl4)</i>	1.21*	1.12	1.23*	1.50*	1.50*
<i>Chemokine (C-C motif) ligand 7 (CCl7)</i>	1.39*	1.29*	1.35*	2.37*	4.32*
<i>Chemokine (C-X-C motif) ligand 11 (CXCl1)</i>	3.05*	2.26*	2.76*	3.20*	2.71*
<i>Chemokine (C-X-C motif) ligand 12 (CXCl2)</i>	1.46*	1.12	1.22*	1.57*	1.42*
<i>Chemokine (C-X-C motif) ligand 5 (CXCl5)</i>	2.52*	2.06*	3.39*	4.32*	3.96*
<i>Chemokine (C-X-C motif) ligand 19 (CXCl9)</i>	1.09	1.29*	1.31*	2.08*	2.91*
<i>Chemokine (C-X-C motif) ligand 10 (CXCl10)</i>	1.00	1.05	1.04	1.04	1.52*
<i>Chemokine (C-X-C motif) ligand 11 (CXCl11)</i>	1.15	1.04	1.05	1.40*	2.63*
<i>Interleukin 1 beta (IL1β)</i>	1.21*	1.16	1.22*	1.48*	1.73*

Gene	Fold change in expression (post-exposure time intervals)					
	1 Week	2 Weeks	4 Weeks	8 Weeks	16 Weeks	
<i>Interleukin 1 receptor antagonist, transcript variant 2 (IL1R2)</i>	1.45*	1.21*	1.21*	2.27*	2.80*	
<i>Resistin like alpha (RETNLA)</i>	2.46*	2.52*	2.42*	3.57*	8.46*	
<i>S100 calcium binding protein A8 (S100A8)</i>	1.26*	1.16	1.42*	2.50*	3.59*	
<i>Triggering receptor expressed on myeloid cells 1 (TREM1)</i>	1.30*	1.08	1.10	1.46*	1.72*	
<i>Triggering receptor expressed on myeloid cells 2 (TREM2)</i>	1.50*	1.28*	1.27	1.63*	1.64*	
<i>Lipocalin 2 (LCN2)</i>	3.24*	3.34*	3.58*	5.53*	5.96*	
<i>Chitinase 3-like 1 (CHI3L1)</i>	1.89*	2.15*	2.17*	2.75*	3.34*	
<i>Secreted phosphoprotein 1 (SPP1)</i>	1.34	1.01	1.12	1.85*	6.27*	
<i>Arachidonate 15-lipoxygenase (ALOX15)</i>	-1.44*	-1.40*	-1.30*	-1.89*	-1.71*	
<b>Tissue remodeling/fibrosis</b>						
<i>Matrix metalloproteinase 8 (MMP8)</i>	1.09	1.13*	1.22*	1.33*	1.50*	
<i>Matrix metalloproteinase 12 (MMP12)</i>	3.78*	3.49*	3.87*	4.34*	4.96*	
<i>Secreted phosphoprotein 1 (SPP1)</i>	1.34	1.01	1.12	1.85*	6.27*	
<i>Haptoglobin (HP)</i>	1.49	1.56*	1.78*	2.11*	2.42*	
<i>Arginase 1 (ARG1)</i>	1.59*	1.29*	1.38*	1.93*	2.28*	
<i>Chemokine (C-X-C motif) ligand 11 (CXCL9)</i>	1.09	1.29*	1.31*	2.08*	2.91*	
<i>Chemokine (C-C motif) ligand 2 (CCL2)</i>	2.36*	1.99*	2.30*	4.28*	6.22*	
<i>Chemokine (C-C motif) ligand 7 (CCL7)</i>	1.39*	1.29*	1.35*	2.37*	4.32*	
<i>Complement component 2 (C2)</i>	1.21*	1.02	1.05	1.19*	1.5*	
<i>Complement component 3 (C3)</i>	2.50*	2.14*	2.26*	2.88*	3.02*	
<i>Complement component 4 binding protein, alpha (C4BPA)</i>	2.14*	2.19*	2.23*	2.90*	3.55*	
<i>Complement component 5 (C5)</i>	1.19*	1.24*	1.31*	1.533*	1.62*	
<i>Complement factor B (CFB)</i>	1.32*	1.18*	1.22*	1.37*	1.5*	
<i>Complement factor 1 (CF1)</i>	1.28	1.47*	1.65*	2.09*	4.18*	
<b>Solute carrier family of genes</b>						
<i>Solute carrier family 26, member 4 (SLC26A4)</i>	4.46*	3.67*	4.10*	6.51*	9.93*	

Gene	Fold change in expression (post-exposure time intervals)				
	1 Week	2 Weeks	4 Weeks	8 Weeks	16 Weeks
<i>Solute carrier family 13, member 2 (SLC13A2)</i>	1.74 <sup>*</sup>	1.51 <sup>*</sup>	1.72 <sup>*</sup>	2.21 <sup>*</sup>	2.50 <sup>*</sup>
<i>Solute carrier family 7, member 7 (SLC7A7)</i>	1.50 <sup>*</sup>	1.43 <sup>*</sup>	1.48 <sup>*</sup>	1.86 <sup>*</sup>	2.33 <sup>*</sup>
<i>Solute carrier family 16, member 3 (SLC16A3)</i>	1.43 <sup>*</sup>	1.23 <sup>*</sup>	1.22 <sup>*</sup>	1.72 <sup>*</sup>	2.02 <sup>*</sup>
<i>Solute carrier family 16, member 11 (SLC16A11)</i>	1.43 <sup>*</sup>	1.58 <sup>*</sup>	1.59 <sup>*</sup>	1.81 <sup>*</sup>	1.88 <sup>*</sup>

Data represent the group means of the silica-exposed rats (*n* = 8) compared with the corresponding time-matched controls (*n* = 4) and were obtained from the microarray analysis results. Some of the genes are listed under more than one category since bioinformatics analysis identified their involvement in multiple categories.

<sup>\*</sup> Statistically significantly different (FDR, *P* < 0.01) compared with the time-matched control samples.

**Table 4**

Correlation co-efficients ( $r^2$  values) for the relationship between pulmonary toxicity and inflammation measurements (LDH, PMN and MCP1) and lung gene expression data in the silica exposed rats

	Correlation co-efficient ( $r^2$ values)		
	LDH	PMN	MCP1
SDEGs	0.80	0.94	1
Biological functions	0.94	0.81	0.85
Canonical pathways	0.86	0.94	0.98
Molecular networks	0.74	0.95	0.98

The toxicity and inflammation measurements were obtained from Table 2 and the gene expression data were obtained from Figs 1 and 3.